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Inhibition of Cholesteryl Ester Transfer Protein Activity by Monoclonal Antibody

Effects on Cholesteryl Ester Formation and Neutral Lipid Mass Transfer in Human Plasma

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Abstract

We have employed a neutralizing monoclonal antibody, prepared against the M_r 74,000 cholesteryl ester transfer protein (CETP), to investigate the regulation of lecithin:cholesterol acyltransferase (LCAT) activity by cholesteryl ester (CE) transfer, and also to determine which lipoproteins are substrates for LCAT in human plasma. The incubation of normolipidemic plasma led to transfer of CE from HDL to VLDL, and of triglycerides from VLDL to LDL and HDL. This net mass transfer of neutral lipids between the lipoproteins was eliminated by the monoclonal antibody. However, CE transfer inhibition had no effect on the rate of plasma cholesterol esterification in plasma incubated from 10 min to 24 h at 37°C. In the absence of CE transfer, HDL and LDL exhibited cholesterol esterification activity, whereas VLDL did not. The rate of CE formation in HDL was three to four times greater than in LDL during the first hour of incubation, but CE formation in HDL decreased after 6–8 h, while that in LDL continued. Thus, (*a*) the M_r 74,000 CETP is responsible for all neutral lipid mass transfer in incubated human plasma, (*b*) the rate of CE formation in plasma is not regulated by CE transfer from HDL to other lipoproteins, and (*c*) HDL is the major initial substrate for LCAT; LDL assumes a more significant role only after prolonged incubation of plasma.

Introduction

Cholesteryl esters (CE)[1] are actively formed and redistributed among lipoproteins in human plasma. Cholesterol is esterified in plasma by the action of lecithin:cholesterol acyltransferase (LCAT, EC 2.3.1.43) (1, 2), then the CE is redistributed among lipoproteins by lipid transfer processes. Nichols et al. (3, 4) first reported the net transfer of CE and triglycerides between lipoproteins in incubated plasma. Using reconstituted lipoproteins, proteins purified from plasma were subsequently shown to promote the reciprocal transfer of triglycerides and CE between triglyceride-rich lipoproteins and HDL particles (5–8). Although the neutral lipid transfer activity of these protein preparations has been shown using isotopic and mass assays in reconstituted systems, there has been no direct evidence linking the activity of a specific lipid transfer protein with the exchange of CE and triglycerides exhibited in whole plasma.

Recently, an M_r 74,000 cholesteryl ester transfer protein (CETP) has been purified from human plasma (9), and used to prepare monoclonal antibodies (10). These monoclonal antibodies were found to inhibit completely the CE and triglyceride transfer activities, and partially inhibit phospholipid transfer activity in human plasma. However, these experiments employed radioassays to measure transfer activities and did not directly demonstrate that mass transfer of neutral lipids in incubated plasma was due only to the M_r 74,000 CETP. One of the CETP monoclonal antibodies, TP2, formerly 5C7 (10), is used in the present study to demonstrate the role of CETP in the mass transfer of neutral lipids in human plasma.

Previous studies have suggested that LCAT activity may be regulated by the rate of CE transfer from HDL to other lipoproteins (11, 12). The addition of purified CETP was found to increase cholesterol esterification in an in vitro system consisting of purified enzyme, lecithin, and sphingomyelin liposomes (CE donor and acceptor particles, respectively) (11). Although the accumulation of newly esterified cholesterol in plasma is accompanied by a decrease in the rate of CE formation (12), there has not been any direct evidence that the transfer of CE out of HDL is the regulatory step for cholesterol esterification. The neutralizing antibody, TP2, thus, provided a tool to determine if CETP regulates LCAT activity in plasma. Moreover, the ability to inhibit CETP activity allowed us to examine the relative importance of LDL and HDL as substrates for the plasma LCAT reaction.

Methods

Materials

[7-^{3}H(*N*)]Cholesterol (11–12 Ci/mmol sp act) was obtained from Amersham Corp. (Arlington Heights, IL). Heparin (porcine instestinal mucosa), diethyl-*p*-nitrophenylphosphate (E600), and BSA were purchased from Sigma Chemical Co. (St. Louis, MO). Precoated TLC silicic acid plates were obtained from Alltech Associates (Deerfield, IL). Scintiverse BD was from Fisher Scientific (Springfield, NJ).

Subjects

Healthy, 12-h fasting human subjects were used as blood donors (Table I). Blood was collected into tubes containing sodium EDTA. Plasma was immediately separated at 4°C by centrifugation and collected. The anti-protease, aprotinin (0.14 TIU/ml plasma), was added to all plasma samples before use in experiments.

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1. *Abbreviations used in this paper:* CE, cholesteryl ester; CETP, cholesteryl ester transfer protein; E600, diethyl-*p*-nitrophenyl phosphate; LCAT, lecithin:cholesterol acyltransferase.

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Inhibition of neutral lipid transfer

Monoclonal antibodies were prepared against partially purified human CETP, as described previously (10). Three monoclonal antibodies were found to be similarly effective in inhibition of neutral lipid transfer. One of these monoclonal antibodies, TP2 (formerly 5C7), was used in the present investigations. Previously (10), nonspecific, monoclonal IgG was found to have no effect on CETP activity in human plasma, and therefore was not used in the current study.

[^{3}H]Cholesterol-labeling of plasma samples

Plasma was labeled with [^{3}H]cholesterol by the injection of [^{3}H]-cholesterol in ethanol (1 μl/ml plasma) into plasma, as described previously (13). Briefly, [^{3}H]cholesterol in ethanol was injected through a 26-gauge needle beneath the surface of stirred plasma on ice. The [^{3}H]cholesterol-labeled plasma (0.2–0.3 μCi/μmol free cholesterol sp act) was placed under a stream of argon for 20 min to remove any traces of ethanol, and left overnight at 4°C before use in experiments. Complete equilibration of the radioactive cholesterol to constant specific activity among lipoproteins was confirmed in preliminary experiments.

Incubation

Plasma or [^{3}H]cholesterol-labeled plasma was incubated at 37°C for 24 h in the absence or presence of monoclonal antibody, TP2 (13.5 μg protein/ml plasma). The volume of the sample incubated varied from 2 to 4 ml. Some samples were also incubated in the presence of 2 mM E600, an LCAT inhibitor. Since LCAT (14) and CETP (15) activities are essentially inactive at 4°C, corresponding samples incubated at 4°C served as controls. Aliquots were collected at selected time intervals, and analyzed as described below.

Assay of CE transfer activity

The CE transfer activity was measured in plasma using a radioassay described previously (16). Plasma (20 μl) with or without TP2 was incubated 2 h at 37°C in the presence of [^{3}H]CE-labeled HDL (3–10 nmol CE) and excess LDL (0.25 μmol CE). The volume was adjusted to 100 μl with Tris-saline (pH 7.4) before incubation. After precipitation of VLDL + LDL by heparin and $MnCl_2$ (17), half of the supernatant volume was removed and counted in a liquid scintillation counter.

*Table I. Profile of Blood Donors Used in the Study**

Subject No.	Sex	Age	Total cholesterol	HDL cholesterol	Triglycerides
		yr		*mg/ml plasma*	
1	M	36	1.45	0.37	0.83
2	F	27	1.93	0.60	0.79
3	M	41	1.73	0.63	0.58
4	M	45	2.63	0.53	0.85
5	M	23	1.39	0.54	0.41
6	F	33	1.20	0.71	0.24
7	M	24	1.96	0.47	0.79
8	M	30	1.84	0.39	1.02
9	M	24	1.24	0.41	0.73
10	F	23	1.67	0.64	0.46
11	M	34	2.09	0.44	1.33
12	M	43	1.95	0.76	0.34
13	M	25	1.75	0.53	0.54
14	F	28	2.13	0.53	0.95
15	F	26	1.68	0.72	0.36
Average			1.78	0.55	0.68
SEM			0.096	0.033	0.079

* 15 subjects were used in the experiment described in Fig. 2. The profile of the subjects includes sex, age, plasma total cholesterol, HDL total cholesterol, and triglycerides. Values of lipids are expressed as mg/ml plasma. The mean values and SEM are also shown.

Assay of LCAT activity

Method 1. Plasma was incubated at 37°C with or without TP2, and LCAT activity was measured as the decline in free cholesterol. Aliquots were removed at specified time intervals, and octuplicate samples (5 μl each) were analyzed for free cholesterol, using an enzymatic determination kit (310–328; Boehringer-Mannheim Diagnostics, Indianapolis, IN). To compensate for the small sample size, the amount of enzymatic reagent was decreased proportionally. Cholesterol calibrators also from Boehringer-Mannheim were used as standards for the assay. LCAT activity is expressed as the amount of CE formed (nmol/ml plasma), calculated from the decline in free cholesterol (mg/ml plasma).

Method 2. Another method used for the assay of LCAT activity was the formation of [^{3}H]CE in [^{3}H]cholesterol-labeled plasma incubated in the presence and absence of TP2. Lipids were extracted from 100-μl aliquots using the Folch extraction (18), dried, and separated by thin-layer chromatography. The dried lipid residue was dissolved in 80–100 μl ether containing cholesterol and cholesteryl oleate as carriers and spotted onto the plate. The developing solvent was hexane/ether/acetic acid (70:30:1, vol/vol/vol). The free cholesterol and CE spots were identified by iodine staining, scraped into vials, and counted in scintillation liquid on a Tri-Carb LS spectrometer (3255; Packard Instruments Inc., Downers Grove, IL). For determination of LCAT activity, the difference between control and experimental values was used to calculate the nanomoles of CE formed per milliliter plasma.

In some experiments using [^{3}H]cholesterol-labeled plasma incubated at 37°C, 400-μl aliquots were treated with heparin (250 U/ml plasma) and $MnCl_2$ (0.09 M, final concentration) to precipitate VLDL and LDL, and obtain the HDL in the supernatant (16). The [^{3}H]-cholesterol and [^{3}H]CE were analyzed similarly, in the HDL supernatant, as well as VLDL plus LDL precipitate, which was redissolved in 400 μl 2 M NaCl.

Separation of lipoprotein fractions

The lipid composition in VLDL, LDL, and HDL from incubated plasma samples were analyzed in some experiments. VLDL was removed from 2-ml incubated plasma samples by ultracentrifugation at $d = 1.006$. Samples were centrifuged 16 h at 4°C in a 50.3 rotor, at 45,000 rpm. Plasma was treated with heparin and $MnCl_2$ as described above. Total cholesterol, free cholesterol, triglycerides, and phospholipids were analyzed in plasma, VLDL, HDL, and $d > 1.006$ fractions. CE was reported as the difference between total and free cholesterol. The amount of lipid in the LDL fraction was calculated as the difference between the amounts of lipid in the $d > 1.006$ and the HDL fractions.

Compositional analysis

Free cholesterol (310–328), total cholesterol (237–574), and triglycerides (877–557) were analyzed using enzymatic kits from Boehringer Mannheim Diagnostics. The triglyceride kit measured triglycerides after conversion of any free glycerol in the sample to an inactive product. To analyze small amounts of sample (5–20 μl), the amount of enzymatic reagent was decreased proportionally in all enzymatic kits. Standards for the assays were also obtained from Boehringer. Phospholipids were measured according to the method of Bartlett (19). The Lowry assay (20) was used to determine protein, using BSA as the standard.

Statistical analysis

Paired *t* test (two-tailed) was used to determine statistical significance. All data are expressed as mean±SEM, unless otherwise indicated.

Results

Effect of TP2 on mass transfer of cholesteryl esters and triglycerides in plasma. The goal of the initial experiments was to determine if the monoclonal antibody, TP2, caused inhibition of neutral lipid transfer in human plasma. Using an isotopic assay, we found inhibition of 99%±6.9% of plasma CE transfer activity, similar to the previous report (10). To evaluate the effect of the monoclonal antibody on net mass transfer of lipids in the absence of CE formation, plasma was incubated at 37°C with an LCAT inhibitor, diethyl-*p*-nitrophenyl phosphate (E600), with and without the CETP neutralizing monoclonal antibody, TP2. The incubation of plasma caused a net gain of CE in VLDL, with a corresponding decrease in HDL, indicating transfer of CE from HDL to VLDL (Table II). There was a concomitant decline in VLDL triglycerides and an increase in LDL and HDL triglycerides, indicating transfer from VLDL to LDL and HDL. Although the rise in LDL triglycerides was not accompanied by a fall in CE, this may have been due to the amount of error associated with the measurement of CE mass in LDL, which was comparable to the expected decrement of LDL CE. There was also a slight increase in VLDL free cholesterol. In contrast, when CETP was inhibited by the addition of TP2, there was no significant change in the CE or triglyceride mass of VLDL, LDL, and HDL. Thus, the M_r 74,000 CETP is responsible for the net mass transfer of CE and triglycerides among the major lipoprotein classes in incubated human plasma.

*Table II. Changes in Free Cholesterol, Cholesteryl Esters, and Triglycerides of Plasma Samples Containing LCAT Inhibitor, E600, and Incubated in the Presence and Absence of Monoclonal Antibody, TP2**

Lipoprotein	4°C Control	− Monoclonal antibody TP2	+ Monoclonal antibody TP2
		mg/ml plasma	
VLDL			
Free cholesterol	0.02 (0.004)	0.04 (0.009)	0.05 (0.008)
Cholesteryl ester	0.06 (0.016)	0.15 (0.038)‡	0.06 (0.010)
Triglycerides	0.29 (0.072)	0.15 (0.040)‡	0.29 (0.072)
LDL			
Free cholesterol	0.29 (0.055)	0.31 (0.055)	0.29 (0.056)
Cholesteryl ester	1.25 (0.273)	1.25 (0.248)	1.29 (0.258)
Triglycerides	0.11 (0.020)	0.30 (0.064)‡	0.12 (0.017)
HDL			
Free cholesterol	0.13 (0.013)	0.12 (0.009)	0.12 (0.011)
Cholesteryl ester	0.69 (0.069)	0.61 (0.062)†	0.69 (0.081)
Triglycerides	0.13 (0.011)	0.18 (0.011)‡	0.12 (0.008)

* Plasma samples (2 ml) were incubated in the presence of 2 mM E600 for 24 h at 4°C (Control), 37°C without (−) and with (+) the monoclonal antibody prepared against CETP, TP2 (13.5 μg protein/ml plasma). VLDL was removed by ultracentrifugation of plasma (2 ml) in a 50.3 rotor at 45,000 rpm, 4°C, for 16 h. HDL fraction was isolated by treatment of plasma with heparin and $MnCl_2$. Lipids were measured in plasma, VLDL, HDL, and $d > 1.006$ fraction. LDL lipid values were calculated by the subtraction of HDL lipid value from that of $d > 1.006$ fraction. Free cholesterol, total cholesterol, and triglycerides were analyzed by enzymatic assays. CE was determined as the difference between total and free cholesterol. Phospholipid was analyzed using the Bartlett method (27). Values are expressed as mean (SEM) from $n = 6$. Statistically significant differences between samples incubated at 4°C or at 37°C (−TP2) are shown. († $P < 0.05$, ‡ $P < 0.005$, as determined by paired t test.)

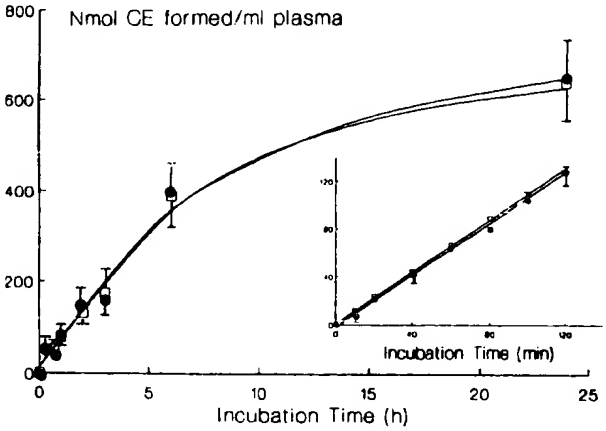

Figure 1. LCAT activity in plasma incubated in the presence or absence of CE transfer. Fresh human plasma (2 ml) obtained from fasting subjects ($n = 7$) was incubated for 24 h at 37°C without (*closed circles*) or with (*open squares*) the TP2 (13.5 μg/ml plasma), a monoclonal antibody prepared against CETP. Aliquots were removed at selected time intervals, and free cholesterol was measured using an enzymatic assay. LCAT activity was measured as the decline in free cholesterol mass, from which nmol CE formed/ml plasma was then calculated. (*Inset*) Some plasma samples ($n = 3$) were labeled with [^{3}H]cholesterol, by the addition of radioactive cholesterol in ethanol to cold, stirred plasma. The labeled samples (0.2–0.3 μCi/μmol free cholesterol sp act) were allowed to equilibrate overnight at 4°C, and then incubated at 37°C without (*closed circles*) or with (*open squares*) the TP2. Aliquots were removed at selected time intervals from 10 min to 2 h. Lipids were extracted, dried, and separated by thin-layer chromatography. The mass of free cholesterol was also analyzed to obtain specific activity, from which amount of cholesterol esterified was calculated. Enzyme activity in both graphs is expressed as nmol CE formed/ml plasma. The mean and SEM values are indicated on the graph.

Effect of inhibition of CETP on the plasma LCAT reaction. The inhibition of CE transfer enabled us to determine if CE transfer affected the formation of CE in whole plasma. Human plasma was incubated for 24 h with or without CETP active, and free cholesterol mass was measured in aliquots removed at selected time intervals. CE formation increased in an approximately linear fashion, but then slowed after 6 h incubation in all plasma samples (Fig. 1). It is notable that the elimination of CE transfer had no effect on the rate of cholesterol esterification, even after 24 h incubation.

Mass analysis of free cholesterol as an indicator of LCAT activity was not sufficiently sensitive to determine the effect of CE transfer on the rate of the LCAT reaction during the first hour of incubation. To measure the rate of CE formation from 10 min to 2 h, some plasma samples were labeled with [^{3}H]-cholesterol, and incubated with or without active CETP. As can be seen in the inset of Fig. 1, the initial rate of cholesterol esterification in plasma was not altered by the inhibition of CE transfer. These results indicate that accumulation of CE in lipoproteins does not influence the rate of the formation of CE in whole plasma, i.e., the transfer of CE is not rate limiting for the LCAT reaction.

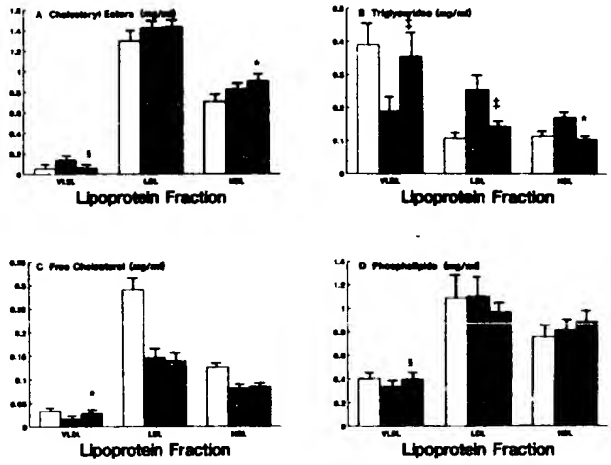

Figure 2. Lipid profile of VLDL, LDL, and HDL from plasma incubated 24 h with and without CETP activity. Plasma samples were incubated 24 h at 4°C (*open bar*), 37°C without (*hatched bar*), and with TP2 (*solid bar*), as described in Table II, with the exception that LCAT was active during incubations. Separation of lipoproteins and subsequent analysis was done as in Table II. The mean and SEM are shown for CE (*A*, $n = 15$), triglycerides (*B*, $n = 15$), free cholesterol (*C*, $n = 15$), and phospholipids (*D*, $n = 6$). Statistically significant differences between samples incubated at 4°C or at 37°C (without TP2) are shown (*$P < 0.001$, ‡$P < 0.025$, or §$P < 0.01$), using paired *t* test.

Effect of lipid transfer inhibition in plasma containing active LCAT. These experiments were performed to determine the effects of CETP activity on lipid distribution in plasma containing active LCAT. Plasma was kept at 4°C (control, *open bar*) or incubated at 37°C, with (*hatched bar*) or without (*solid bar*) CETP activity; the symbols above the bars indicate significant differences between samples incubated with and without CETP activity (Fig. 2). With CETP active, there were net increases of CE mass in VLDL, LDL, and HDL, reflecting the combined activities of LCAT and CETP (Fig. 2 *A*). When transfer activity was inhibited, and only cholesterol esterification was active, CE accumulated in LDL, as well as HDL, but not in VLDL. This indicates that the CETP transfers CE from HDL to VLDL, as in the LCAT-inhibited state (Table II). The results also show that since the increase in LDL CE in plasma without CETP activity cannot be due to transfer, LDL also serves as a direct substrate for LCAT. Again, triglyceride transfer from VLDL to LDL and HDL was abolished by inhibition of CETP (Fig. 2 *B*).

Since LCAT activity was not associated with VLDL, the accumulation of CE in VLDL was due solely to transfer. We found no significant difference between net mass transfer of CE to VLDL in plasma samples incubated with inactive LCAT (0.09±0.066 mg CE transferred/ml plasma per 24 h, Table II), and that in corresponding samples (same subjects) incubated with active LCAT (0.073±0.055 mg CE transferred/ml per 24 h, Fig. 2 *A*). This suggests that in normolipidemic plasma incubated for 24 h, neutral lipid transfer is not influenced by LCAT action.

After incubation of plasma at 37°C, the amount of free cholesterol declined in all lipoprotein classes, as expected for continued LCAT action (Fig. 2 *C*). The decrease was greatest in LDL, demonstrating that LDL provides the major source of free cholesterol for the LCAT reaction, as previously described (12). VLDL free cholesterol did decline slightly when CETP was active. VLDL phospholipid was also decreased with CETP active (Fig. 2 *D*). However, there was no decline in either free cholesterol or phospholipid in VLDL from plasma incubated with CETP inactive, consistent with the CETP playing a role in transfer of phospholipids from VLDL to other lipoproteins. The changes in free cholesterol in VLDL may have been secondary to the changes in VLDL phospholipid, since phospholipid enrichment of lipoproteins in plasma has been shown to be accompanied by an enrichment in unesterified cholesterol (21).

Sites of cholesterol esterification in human plasma. The use of TP2 to inhibit CE transfer also allowed us to determine the sites of cholesterol esterification in plasma. The results of the 24-h incubation (Fig. 2) indicated that both LDL and HDL may be important substrates for LCAT.[2] To determine if LDL was an important substrate for LCAT over the entire 24-h time period, CE formation in lipoproteins was followed over time by measuring the accumulation of esterified [^{3}H]cholesterol in the HDL and VLDL + LDL fractions. With CETP inactive, any CE accumulation in the lipoprotein fractions was due to action of LCAT on that specific fraction. It was observed previously (Fig. 2) that there was no CE accumulation in VLDL over time, so any increase of CE in the VLDL + LDL fraction was solely in LDL. Although the amount of esterified [^{3}H]-cholesterol increased immediately in both HDL and LDL fractions in plasma without CETP activity, the rate of cholesterol esterification was three to four times greater on HDL than on LDL during the first 2 h of incubation (Fig. 3 *A*). Upon further incubation, the rate of CE formation in the HDL began to decline after 4–6 h incubation, while that of LDL continued to increase. With CETP active, a larger amount of [^{3}H]CE appeared in LDL and VLDL, reflecting transfer from site of formation in HDL to CE acceptor lipoproteins (Fig. 3 *B*).

Correlational analyses: effect of lipoprotein mass ratios on LCAT and lipid transfer activities. A significant correlation was found between the accumulation of CE in LDL after 24 h incubation, when CETP was inhibited, and LDL CE/HDL CE ($r = 0.621$, $P < 0.005$), suggesting that distribution of LCAT activity in incubated plasma can be determined by the relative masses of LDL and HDL. Accumulation of CE in HDL demonstrated no significant correlation with any of the variables measured.

Plasma triglyceride levels correlated inversely with HDL CE ($r = -0.797$, $P < 0.001$), which agrees with previous reports (24–26). With CETP active, strong positive correlations were obtained between transfer of CE to VLDL and the following: VLDL triglycerides (Fig. 4, $r = 0.948$, $P < 0.001$), VLDL triglycerides/HDL CE ($r = 0.916$, $P < 0.001$), and VLDL triglycerides/LDL CE + HDL CE ($r = 0.861$, $P < 0.001$). When the CETP was inactive, there was no longer any correlation

2. It is conceivable that the presence of the TP2 may have induced association of LCAT, along with CETP, with LDL. This, in turn, would result in increased CE formation in the LDL fraction. To test this, LCAT activity, or formation of [^{3}H]cholesteryl esters, was measured in lipoproteins fractions of [^{3}H]cholesterol-labeled plasma, isolated using a Sepharose CL-6B column, as described previously (22). The majority of LCAT activity remained on HDL, similar to that previously described (23), and the presence of the TP2 in plasma did not appear to shift LCAT onto LDL (data not shown).

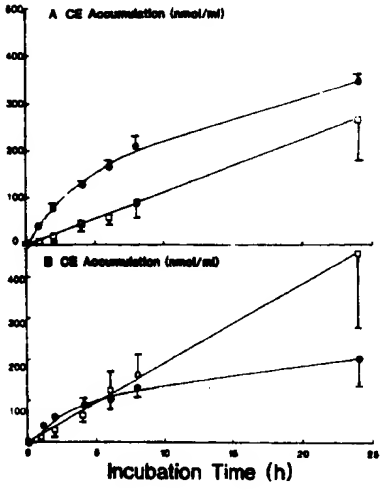

Figure 3. Effect of inhibition of CE transfer on formation of CE in VLDL + LDL, and HDL fractions. [^{3}H]-Cholesterol-labeled plasma (n = 3) was incubated in the presence (*A*) and absence of MAb (*B*), as described in Fig. 1. Aliquots were removed during the incubation and treated with heparin and $MnCl_2$ to precipitate VLDL + LDL. The amount of esterified cholesterol in the HDL (*closed circles*) and VLDL + LDL (*open squares*) was measured as described in Fig. 1.

between CE transfer to VLDL and the lipoprotein mass ratios (Fig. 4, *open squares*). Similar relationships between transfer of triglycerides from VLDL and VLDL mass was observed. This indicates that the level of VLDL triglycerides is the major determinant of the extent of CETP-mediated transfer of neutral lipids among HDL and VLDL in incubated plasma.

Discussion

The recent development of neutralizing monoclonal antibodies to the CETP has allowed us to explore the functions of CETP in human plasma. There were three new findings in the present study. First, the M_r 74,000 CETP is responsible for the net mass transfer of CE from HDL to VLDL, and of triglycerides from VLDL to LDL and HDL in incubated plasma. Secondly, CETP inhibition reveals that the rate of CE formation in HDL is initially rapid, but then declines, whereas that of LDL is slower, but constant throughout the 24-h incubation

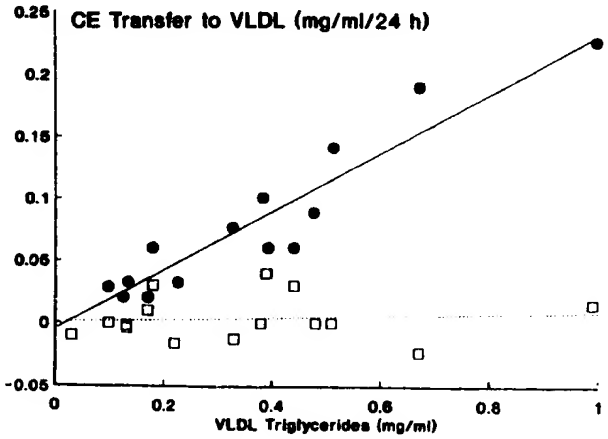

Figure 4. Relationship of CE transfer and VLDL triglycerides. A scatter plot is shown of the transfer of CE to VLDL in the presence (*closed circles*) or absence (*open squares*) of CETP activity (from Fig. 2) versus VLDL triglycerides of the original plasma sample.

period. Consequently, HDL is the major initial substrate for LCAT, but LDL assumes an increasingly important role with continued incubation. Finally, the LCAT reaction in plasma is not regulated by neutral lipid transfer between the lipoproteins.

The CETP monoclonal antibody blocked the shift of neutral lipids between lipoproteins in incubated plasma, a process originally described by Nichols et al. (3, 4). Previous studies have identified plasma protein fractions that promoted exchange of CE and triglycerides (7–9, 27), and even net transfer of neutral lipids (5, 8, 9, 28) in reconstituted lipoprotein systems. However, the specific role of these transfer protein(s) in whole plasma was uncertain. Our results show that there is no net movement of neutral lipids due to either collisional or diffusional processes, or other lipid transfer proteins. The M_r 74,000 CETP protein was directly responsible for the net transfer of CE and triglycerides among plasma lipoproteins.

While CETP may mediate the transfer of neutral lipids between VLDL and HDL, our results confirmed that the mass of CE acceptor lipoproteins is a major determinant of the amount of net neutral lipid transfer (Fig. 4), as previously postulated (29). Along with the observations made in cross-sectional studies, in which HDL CE was inversely correlated with plasma or VLDL triglycerides (24–26, 30, 31), the results suggest that the mass of triglyceride rich acceptor lipoproteins influences the rate of CE transfer from HDL, and thereby, in part, determines HDL CE mass, in vivo. Although other factors such as lipolysis (32), CETP mass (13), or free cholesterol mass (33) may influence the rate of CE transfer, it is evident that in the 15 normolipidemic subjects of this study, triglyceride mass is a strong predictor of net neutral lipid transfer in incubated plasma.

Since the inhibition of LCAT activity did not affect the amount of CE transferred to VLDL from HDL, cholesterol esterification may not influence CE transfer in normolipidemic plasma incubated for 24 h. A similar lack of effect of LCAT on the initial rate of CE transfer had also been reported (12). In contrast, CE transfer from VLDL to HDL was more pronounced with active LCAT in an earlier study (34). However, this was observed under hypertriglyceridemic conditions in a recombinant system of high levels of VLDL from an LCAT-deficient patient and HDL from a normal subject. In addition, this study used *p*-chloromercuri-phenyl sulfonate, which is now known to be a neutral lipid transfer inhibitor (35), as well as an LCAT inhibitor. Thus, the slower transfer in the LCAT inhibited plasma may have been due to a direct effect of *p*-chloromercuriphenyl sulfonate on CETP. Our results suggest that in normolipidemic plasma, LCAT does not play an important role in determining CE transfer rate.

The relative importance of the different lipoproteins as substrates for LCAT in plasma has been somewhat controversial. The ability to inhibit neutral lipid transfer in human plasma permitted a new and direct approach to investigate the question of which lipoproteins accumulate CE and therefore serve as the native substrate for LCAT in human plasma. A dominant role of HDL and a lesser role of LDL as LCAT substrate has been demonstrated in earlier studies, which used ultracentrifugally isolated lipoproteins (36, 37). However, a recent study using pig plasma, which lacks neutral lipid transfer activity, has suggested that LDL is the major substrate for LCAT (38). The conclusion that the majority of newly formed CE was on pig LDL was based on data largely derived

from plasma incubated for long periods of time. The results obtained in the present study clearly show that HDL is the primary substrate for the initial LCAT reaction in whole human plasma. LDL is a minor initial substrate, but becomes more important with continued incubation of plasma.

An unexpected finding in the present study was that neither the initial rate of CE formation, nor the accumulation of CE over the 24-h incubation was affected by the blockade of neutral lipid transfer in incubated plasma. Previous investigations suggested that CE transfer between lipoproteins is limiting for cholesterol esterification, based on in vitro incubations using vesicular lipoprotein particles (11). In whole plasma, native, spherical lipoproteins may have greater flexibility to accomodate CE, products of the LCAT reaction, without adversely influencing LCAT activity. Also, we showed that LDL can assume a significant role as an LCAT substrate when CE formation becomes limited in HDL (i.e., after 6–8 h incubation). This versatility of LDL as an alternative site for LCAT reaction may allow the total plasma LCAT activity to remain the same, even during prolonged incubation of plasma with CETP inactive. The decline in HDL CE formation after 6–8 h incubation (Fig. 3) may have reflected product inhibition in HDL, and/or depletion of available substrates in HDL, leading to the subsequent shift of cholesterol esterification from HDL to LDL.

Our findings have implications for the role of CETP in reverse cholesterol transport, a pathway postulated to be antiatherogenic. In one version of this theory, cholesterol diffuses from tissues into HDL, is esterified by LCAT action, and then transferred to VLDL for ultimate removal by the liver (39). In tissue culture experiments, plasma LCAT activity promotes removal of free cholesterol and CE from cells (40). If the CE transfer process was able to drive the LCAT reaction, this would imply indirect regulation of tissue cholesterol removal by CETP. The lack of effect of CETP on cholesterol esterification indicates that this mode of regulation is unlikely to occur in human plasma. However, CETP does influence the transfer of CE from HDL to triglyceride-rich lipoproteins, and this may ultimately influence the rate of transfer of CE from plasma to the liver.

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